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ORIGINAL ARTICLE

A suprachiasmatic-independent circadian clock(s) in the habenula is affected by *Per* gene mutations and housing light conditions in mice

Nora L. Salaberry[1] · Hélène Hamm[1] · Marie-Paule Felder-Schmittbuhl[1] · Jorge Mendoza[1]

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Abstract

For many years, the suprachiasmatic nucleus (SCN) was considered as the unique circadian pacemaker in the mammalian brain. Currently, it is known that other brain areas are able to oscillate in a circadian manner. However, many of them are dependent on, or synchronized by, the SCN. The Habenula (Hb), localized in the epithalamus, is a key nucleus for the regulation of monoamine activity (dopamine, serotonin) and presents circadian features; nonetheless, the clock properties of the Hb are not fully described. Here, we report, first, circadian expression of clock genes in the lateral habenula (LHb) under constant darkness (DD) condition in wild-type mice which is disturbed in double *Per1*$^{-/-}$-*Per2*^{Brdm1} clock-mutant mice. Second, using *Per2::luciferase* transgenic mice, we observed a self-sustained oscillatory ability (PER2::LUCIFERASE bioluminescence rhythmicity) in the rostral and caudal part of the Hb of arrhythmic SCN-ablated animals. Finally, in *Per2::luciferase* mice exposed to different lighting conditions (light-dark, constant darkness or constant light), the period or amplitude of PER2 oscillations, in both the rostral and caudal Hb, were similar. However, under DD condition or from SCN-lesioned mice, these two Hb regions were out of phase, suggesting an uncoupling of two putative Hb oscillators. Altogether, these results suggest that an autonomous clock in the rostral and caudal part of the Hb requires integrity of circadian genes to tick, and light information or SCN innervation to keep synchrony. The relevance of the Hb timing might reside in the regulation of circadian functions linked to motivational (reward) and emotional (mood) processes.

Keywords Circadian · Habenula · Per2 luciferase · Suprachiasmatic · Clock genes

Introduction

In humans, and in other species, circadian rhythms consist of behavioral, physiological and molecular changes that follow a roughly 24-h-period and are sustained in constant environmental conditions (Mohawk et al. 2012). Most, if not all, of these rhythms are under the control of the principal clock localized in the hypothalamus: the suprachiasmatic nucleus (SCN; Welsh et al. 2010). The ability of the SCN circadian clock to maintain a rhythmic self-sustained activity bases on a molecular clockwork involving clock genes such as *Clock, Bmal1, Per1-3, Cry1-2*, and *Rev-Erbα*, regulating each other in the form of positive and negative loops (Takahashi et al. 2008). Clock gene expression is not limited to the SCN and has been described in other extra-SCN circadian pacemakers such as the olfactory bulb, the retina and the lateral habenula (Granados-Fuentes et al. 2004; Guilding and Piggins 2007; Ruan et al. 2008).

The habenula (Hb) is a small brain structure localized in the epithalamus (formed by the Hb and pineal gland) and conserved amongst vertebrates (Concha and Wilson 2001). Its lateral part emerged as a key nucleus for dopamine and serotonin control (Christoph et al. 1986; Lecourtier and Kelly 2007; Matsumoto and Hikosaka 2007). The lateral Hb (LHb) receives various inputs from the forebrain such as the lateral septum, preoptic area, entopeduncular nucleus and prefrontal cortex (Zahm and Root 2017). It projects directly and indirectly, via the rostromedial tegmental nucleus (RMTg), to monoaminergic nuclei like the ventral tegmental area (VTA), the *substantia nigra pars compacta* and the raphe nuclei (Sutherland 1982; Lecourtier and Kelly

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✉ Jorge Mendoza
jmendoza@inci-cnrs.unistra.fr

[1] Institute of Cellular and Integrative Neurosciences, CNRS UPR-3212, 5 rue Blaise Pascal, 67000 Strasbourg, France

2007; Brinschwitz et al. 2010; Lammel et al. 2012). Due to its diverse connections, the LHb is involved in many brain processes such as reward, aversion, maternal behavior, pain, anxiety, memory and sleep (Sutherland 1982; Bianco and Wilson 2009; Hikosaka 2010; Aizawa et al. 2013; Mathis et al. 2015). Hence, the LHb acts as a relay which integrates an extensive amount of information from various areas linked to behavior or cognitive functions, to define the most appropriate behavioral strategy.

LHb neurons fire rhythmically and show circadian variations in the expression of the PER2 clock protein ex vivo (Zhao and Rusak 2005; Guilding et al. 2010). Unlike the SCN, the molecular machinery in the Hb is not totally characterized. The expression of some clock genes and proteins has been reported in the LHb in rats and mice (Shieh 2003; Christiansen et al. 2016); but, only the expression of BMAL1, CLOCK and PER2 proteins showed daily rhythms in the mouse LHb in a light-dark cycle (Wyse and Coogan 2010; Zhao et al. 2015). However, to what extent clock genes are necessary to keep circadian oscillations in the LHb remains to be determined to our knowledge. The LHb receives direct or indirect inputs from structures involved in circadian regulation such as the retina and SCN (Qu et al. 1996; Zhao and Rusak 2005; Zhang et al. 2009). Despite good evidence for circadian activity in the LHb (Guilding et al. 2010), its level of SCN-dependence is still unknown. Hints for direct SCN projection to the LHb can be seen through the presence of prokineticin 2 fibers (Zhang et al. 2009). The SCN might also project to the LHb indirectly via the lateral hypothalamus, lateral septum and ventral posterior nucleus, but these connections remain to be confirmed (Guilding and Piggins 2007; Poller et al. 2013; Hernández et al. 2015). In addition, the rat and mouse LHb responds to retinal photo-stimulation, and in *zebrafish* circadian behavior is driven by blue light, a signal transmitted by the retina through a thalamo-Hb pathway (Zhao and Rusak 2005; Dreosti et al. 2014; Sakhi et al. 2014b; Shuboni et al. 2015; Cheng et al. 2017; Lin and Jesuthasan 2017); hence, LHb cells may be synchronized by the solar day and use this light information to regulate behavior. In mammals, the anatomical link between the retina and the LHb has been reported to be in an indirect way since retina fibers do not arrive specifically to the LHb but rather to the peri-habenular region (PHb) in the brain of different rodent species (Qu et al. 1996; Reuss and Decker 1997; Hattar et al. 2006; Fernandez et al. 2018). However, other putative nuclei such as the lateral preoptical area and lateral hypothalamus might be involved (Hattar et al. 2006; Zahm and Root 2017).

In the present study, we first investigated the circadian pattern of clock gene expression by in situ hybridization (mRNA) in the LHb of wild-type (WT) and arrhythmic double $Per1^{-/-}Per2^{Brdm1}$-mutant mice housed under constant darkness condition. Then, to decipher whether the Hb contains a clock independent from the master SCN pacemaker, we used a luciferase reporter to follow PER2 protein expression in Hb explants of intact or SCN-lesioned animals. Finally, the effects of different lighting conditions (light-dark, constant darkness and constant light) on the Hb clockwork were investigated.

Materials and methods

Animals and housing

WT and double $Per1^{-/-}Per2^{Brdm1}$ mutant (Zheng et al. 1999, 2001) male mice on a mixed C57BL/6JxSV129 background ($n=70$; 12–16-week-old) used for the first experiment were housed in groups of 3 or 4 individuals per cage. C57BL/6J homozygous *Period2::luciferase* ($Per2^{Luc}$ or PER2::LUC) knock-in mice (initially from Jackson Laboratories, Bar Harbor, ME, USA; Yoo et al. 2004; $n=52$; 10–13 weeks old at the end of the studies) were obtained from our breeding colony in Strasbourg (Chronobiotron platform, UMS3415, CNRS, University of Strasbourg, France). All mice had access to ad libitum food (UAR, Epinay sur Orge, France) and water, and were housed in light-proof ventilated rooms, under 12 h of white light (150 lx) and 12 h of darkness cycle (LD12:12; lights on at 8:00 A.M.). Dim red light was permanently on (< 5 lx at cage level in the dark). Experiments were performed in accordance with rules from the French National Law and the European Committee Council Directive of September 22, 2010 (2010/63/UE). Experimental protocols were approved by the Regional Ethical Committee of Strasbourg for Animal Experimentation (CREMEAS), and the French Ministry of Higher Education and Research (Approval #2015021811138196).

Experimental design

Experiment 1: circadian clock gene expression in the LHb of WT and double $Per1^{-/-}$-$Per2^{Brdm1}$ mutant mice

To evaluate whether clock genes are expressed in the LHb in a circadian manner, both WT ($n=35$) and double $Per1^{-/-}$-$Per2^{Brdm1}$-mutant ($n=35$) mice were killed under a constant and complete darkness (DD) condition using night vision goggles (ATN NVG7, ATN-Optics, Chorges, France) at eight temporal points: every 3 h from the projected-*Zeitgeber* Time 13 (pZT13; ZT13 represents 1 h after lights off (ZT12) of the last day on LD condition as a phase reference time point) until p-ZT10 of the next day. Therefore, animals were first housed in a LD cycle (1212 h), then transferred to DD condition 24 h before the beginning of the sacrifice ($n=3$–5 per time point). Brains were rapidly removed, frozen on dry ice and stored at −80 °C until use for hybridization.

Experiment 2: circadian rhythms of PER2::LUC bioluminescence in the Hb of SCN-lesioned mice

To investigate the self-sustainability of the Hb clock, we performed bilateral electrolytic SCN lesions on *Per2*^{Luc}-transgenic mice (SCNX; $n = 14$). Mice were placed for 1 week in LD condition to recover from surgery. Then, to avoid any masking effect by light, animals were placed in DD condition for 2–3 weeks under dim red light (5 lx). Locomotor activity was recorded to evaluate SCN lesions. Intact mice (SHAM, $n = 9$) were killed during the subjective day (around CT6-9), and SCNX animals at the geographical time 13-16 h. Brains were rapidly removed and dissected at the level of the Hb. Explants were prepared for bioluminescence recordings.

Experiment 3: circadian rhythms of PER2::LUC bioluminescence in the mouse Hb under different lighting conditions

To unravel the possible effects of lighting conditions on the Hb clock, *Per2*^{Luc} mice were divided into three different lighting groups: LD12:12 (control animals; $n = 14$), DD ($n = 11$) and constant light condition (LL, 150–200 lx at the level of the cage, $n = 12$). All animals were in individual cages, and locomotor activity was recorded. For the DD group, we analyzed data from mice of the SHAM group of experiment 2 to reduce the number of animals used in the whole study. For the LL group, after 1–2 weeks in LD condition, mice were exposed for 5–6 weeks to constant white light. Animals were killed during the day for LD (around ZT6-9), subjective day for DD (CT6-9) or at the geographical time 13–16 h for LL groups, and brain explants were prepared for bioluminescence recordings.

Non-radioactive in situ hybridization

Eighteen-micrometer coronal sections containing the Hb were cut with cryostat at − 20 °C according to mouse brain stereotaxic atlas (Paxinos and Franklin 2001), and directly mounted on slides. Digoxigenin-labeled RNA probes were synthesized from linearized plasmids according to the protocol of the manufacturer, using specific RNA-labeling mix (11277073910, Roche, Bâle, Switzerland). *mClock* plasmid was a gift of Prof. J. S. Takahashi (Northwestern University, Evanston, USA; Tournier et al. 2003). *mPer2* and *mCry2* probes were generated from plasmids kindly provided by Dr. H. Okamura (University of Kobe, Kobe, Japan). *rRev-Erbα* probe was kindly provided by Dr H. Dardente (University of Tours, France). In situ hybridization was performed according to previous studies (Talbi et al. 2016). Sections were fixed in 4% phosphate-buffered paraformaldehyde (PFA) for 10 min, rinsed in phosphate-buffered saline (PBS) and then acetylated twice for 10 min in 1.2% triethanolamine, 0.25% acetic anhydride. Sections were then rinsed in PBS and equilibrated in 5X sodium saline citrate (SSC), 0.05% Tween-20 (Tw) before hybridization. The sections were incubated for 40 h at 62 °C with 100 ng/mL (*rRevErbα*) or 200 ng/mL (*mClock, mCry2, mPer2*) labeled anti-sense probes in the hybridization buffer (50% formamide, 5X SSC, 5X Denhardt's, 1 mg/mL of denatured salmon sperm DNA, 0.1% Tw and 0.04% diethylpyrocarbonate). After hybridization, the probe was washed off in 5X SSC + 0.05% Tw at room temperature. Stringency rinses were performed for 6 × 10 min in 0.1X SSC at 72 °C. Sections were then exposed for 1 h in blocking buffer (0.5% Blocking Reagent [Roche], 100 mM TRIS, 150 mM NaCl, 5 mM $MgCl_2$, 0.05% Tw, 0.02% NaN_3, pH 7.5). Sections were incubated overnight with alkaline phosphatase-labeled anti-digoxigenin antibodies (11093274910, Roche) at the concentration of 1:10 000 for *rRev-Erbα, mPer2* and 1:5 000 for *mClock, mCry2* in 100 mM TRIS, 150 mM NaCl, 5 mM $MgCl_2$, pH 7.5. Phosphatase activity was detected with nitroblue tetrazolium (400 µM, 10,202,610, Fisher Scientific, Waltham, MA, USA) and 5-bromo-4-chloro-3-indolyl phosphate (380 µM, R0821, Fisher Scientific) in 100 mM TRIS, 100 mM NaCl, 5 mM $MgCl_2$ (pH 9.5).

Cell counting and analysis

Brain images were taken with a ProRes® CFcoolJenoptik camera on a Zeiss AxioImager microscope, standardizing all lighting parameters on the microscope and the camera software to ensure consistent stable lighting throughout the image capture, and stored on a PC. Digoxigenin-positively labeled cells were counted using ImageJ (NIH) software. Positive cells were counted in the whole LHb (Four sections/animal, −1.82 to −1.46 mm from Bregma), based on the atlas of Paxinos and Franklin (2001). Data are presented as the mean number of immunopositive cells per section ± SEM. The circadian profiles of gene expression were fitted by a non-linear least-squares cosinor regression analysis (Refinetti et al. 2007) using the SigmaPlot Software (Systat Software Inc., version 12.5). The fitting equation is:

$$y = A + B\cos\left(2\pi\frac{x - C}{24}\right)$$

where y is the number of positively labeled cells, A the mesor (Midline Estimating Statistic Of Rhythm) or the mean level of the oscillation, B the amplitude of the oscillation, C the acrophase (time when the oscillation peaks), x the time (h), and the period is set at 24 h. Only significant best-fit parameters ($p < 0.05$) were included in this study if the residues of the fit respected the analysis of variance (ANOVA) rules.

Locomotor activity recordings

General locomotor activity was measured using infrared detectors placed above the cage and linked to an automated recording system (CAMS, Circadian Activity Monitoring System, Lyon, France). Data were recorded in 5-min bins and plotted as actograms. Clocklab software (Actimetrics, Wilmette, IL, USA) was used to determine the period of circadian behavior (χ^2 periodograms) of each animal under different experimental conditions during intervals of 10 days for LD and LL conditions and 20 days for SHAM and SCNX animals. Data of mice from the SCNX group which showed rhythmic locomotor activity were removed from the study ($n = 5$).

SCN lesions

Per2^{Luc} mice were anaesthetized with 1–3% isoflurane, with subcutaneous injection of buprenorphine (0.05 mg/Kg) as pre- peri- and post-analgesia and with subcutaneous injection of lidocaine (5 mg/Kg; Xylovet, Ceva Santé Animal, Libourne, France) as local analgesia. The animals were placed into a stereotaxic apparatus (David Kopf Instrument, Tujunga, CA, USA). Current (1 mA, 20 s) was applied bilaterally through a stainless steel electrode (300 µm diameter) at the level of the SCN following the coordinates + 0.5 mm (anteriorposterior axis); ±0.2 mm (medio-lateral axis); 5.7 mm (dorso-ventral axis) according to Paxinos and Franklin (2001). SHAM control animals underwent the same surgery procedure without electrical current. For 3 days, mice received an analgesic solution (1 mL/Kg of body weight; Meloxicam, Boehringer, Ingelheim, Germany) in water bottles. For histological verification of SCN lesions, 30 µm coronal sections at the SCN level of SHAM or SCNX mice were fixed with 4% PFA and cut with cryostat at −20 °C according to mouse brain stereotaxic atlas (Paxinos and Franklin 2001), and then mounted on slides. Sections were stained with Cresyl Violet 0.1% for 10 min, then rinsed with distilled water for 2 × 5 min, and dehydrated with increased ethanol concentration (70% for 5 min, 95% for 5 min, 100% for 2 × 5 min), and finally in toluene for 2 × 5 min. Slides were coverslipped with Eukitt® mounting medium. SCNX animals with intact SCN were excluded from the analysis and were the same that kept rhythmic locomotor activity ($n = 5$).

Tissue culture for ex vivo bioluminescence

PER2::LUC bioluminescence recordings were performed using a LumiCycle photon-counting photomultiplier (Actimetrics). Animals were killed by cervical dislocation and brains were rapidly removed and placed in ice-cold 1X Hank's balanced salt solution (HBSS; 10X, H1641, Sigma, Saint-Louis, MO, USA) containing sodium bicarbonate (0.035%, S8761, Sigma), HEPES (10 mM, H0887, Sigma) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, P433, Sigma). Coronal sections (500 µm) of the SCN region and the dorsal thalamus containing the Hb were made using a vibratome (MA752 Campden Instrument, Loughborough, UK) in which the medium was kept cold by 1X HBSS ice cubes. A pair of scalpels was used to cut the brain regions of interest into small sections (1.0 mm^2) according to mouse brain stereotaxic atlas (Paxinos and Franklin 2001). The Hb, 1.2 mm long, was separated into two pieces: rostral and caudal, due to their different innervations and their difference in lateral and medial proportion (Fig. 4c and Fig. S1a; Paxinos and Franklin 2001; Hernández et al. 2015). Then, tissues were cultured on Millicell® culture membranes (PICMORG 50, Millipore, Billerica, MA, USA) in a 35-mm Nunc™ cell culture dish (10654161, Fisher Scientific) with 1 mL Dulbecco's modified Eagle's medium (DMEM; D2902, Sigma) supplemented with D (+) glucose (0.35%, G7021, Sigma), sodium bicarbonate (0.035%), HEPES (10 mM), B27 (2%, 17504–044, Invitrogen, Carlsbad, CA, USA), antibiotics (25 U/mL penicillin and 25 µg/mL streptomycin), and beetle luciferin (0.1 mM, E1602, Promega, Madison, WI, USA). Culture dishes were sealed with high-vacuum grease (Dow corning®, Wiesbaden, Germany) and placed for recording into the LumiCycle device maintained at 37 °C within an incubator. Samples included both the lateral and medial habenula, thus we refer to as the habenular complex (Hb). In the Hb dorsomedial border, there is a layer of ependymal cells which show strong PER2::LUC oscillations (Fig. S1b; Guilding et al. 2010). Thus, to avoid an overexpressed and masked PER2 expression in Hb explants, the ependymal layer was removed from each Hb sample.

Bioluminescence recording and analysis

Bioluminescence was recorded for 112 s (every 15 min) for at least 5 days. Waveforms of rhythmic bioluminescence emission were analyzed with the LumiCycle Analysis software (Actimetrics). Data were smoothed with a 5 h running average method and detrended using a 24 h running average subtraction (LumiCycle Analysis software; Actimetrics). We chose as time reference ($t = 0$) midnight before the start of the experiment: in this context, $t = 1$ day means midnight after the start of the experiment. The period and first peak amplitude were determined within a 3-day time window (from around $t = 2$ to $t = 5$ days) using the LM sin fit damped method. The phase was determined as the midpoint of the first peak, which occurred between 24 and 48 h of culture. Then, we calculated the absolute difference between Hbr and Hbc phases (Δ phase$_{(Hbr-Hbc)}$) by subtracting the Hbc phase from that of Hbr; the more the value will be close to 0, the higher the two areas will be close in phase. Tissue

samples that did not survive (bioluminescence activity as low as for an empty dish) were excluded from the analysis. The threshold for goodness of fit was set at 70%; when below, data were considered arrhythmic. Data are presented in hours for the period and phase, and as relative bioluminescence activity in counts per second (cps) for the amplitude.

Statistical analysis

All results are indicated as mean ± SEM except for the phases where only the median is shown. Graphs were made with SigmaPlot (Systat Software Inc., version 12.5, Chicago, IL, USA) or GraphPad Prism software (GraphPad Software, Inc., version 6, La Jolla, CA, USA) and all the figures were made with Adobe Illustrator CS6 (Adobe systems, San Jose, CA, USA). Data were analyzed by two-way ANOVA, ANOVA on ranks or unpaired Student's *t* test using SigmaPlot. Amplitude values from Hb samples were normalized after log transformation. Significant differences were analyzed with Holm–Sidak-corrected or with Tukey-corrected (only for ANOVA on rank analysis) post hoc comparisons. The homogeneity of variance of Δ values was tested with Levene's test using Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA). α level was set at 0.05 for all experiments.

Results

Clock gene expression in the LHb is altered in double *Per1*$^{-/-}$–*Per2*^{Brdm1}-mutant mice

Rhythmic expression of *Clock, Cry2* and *Rev-Erbα* mRNA was observed in the LHb under DD condition (Fig. 1a). LHb cells expressed *Per2* gene in a narrow time window around p-ZT10 (late resting state) but the fit of cosinor regression was not optimal enough to indicate a significant rhythmic expression (Fig. 1b; Table 1). Circadian rhythms of *Rev-Erbα* and *Cry2* peaked during the early and middle subjective active phase, respectively (Fig. 1b). Interestingly, the circadian oscillation of the *Clock* gene peaked during the late subjective active phase, in antiphase with the expression of *Rev-Erbα* (Fig. 1b; Table 1). However, in double *Per1*$^{-/-}$–*Per2*^{Brdm1}-mutant mice, *Clock* and *Rev-Erbα* genes lost rhythmicity with consistency (but different from zero) for *Rev-Erbα* and wide variability for

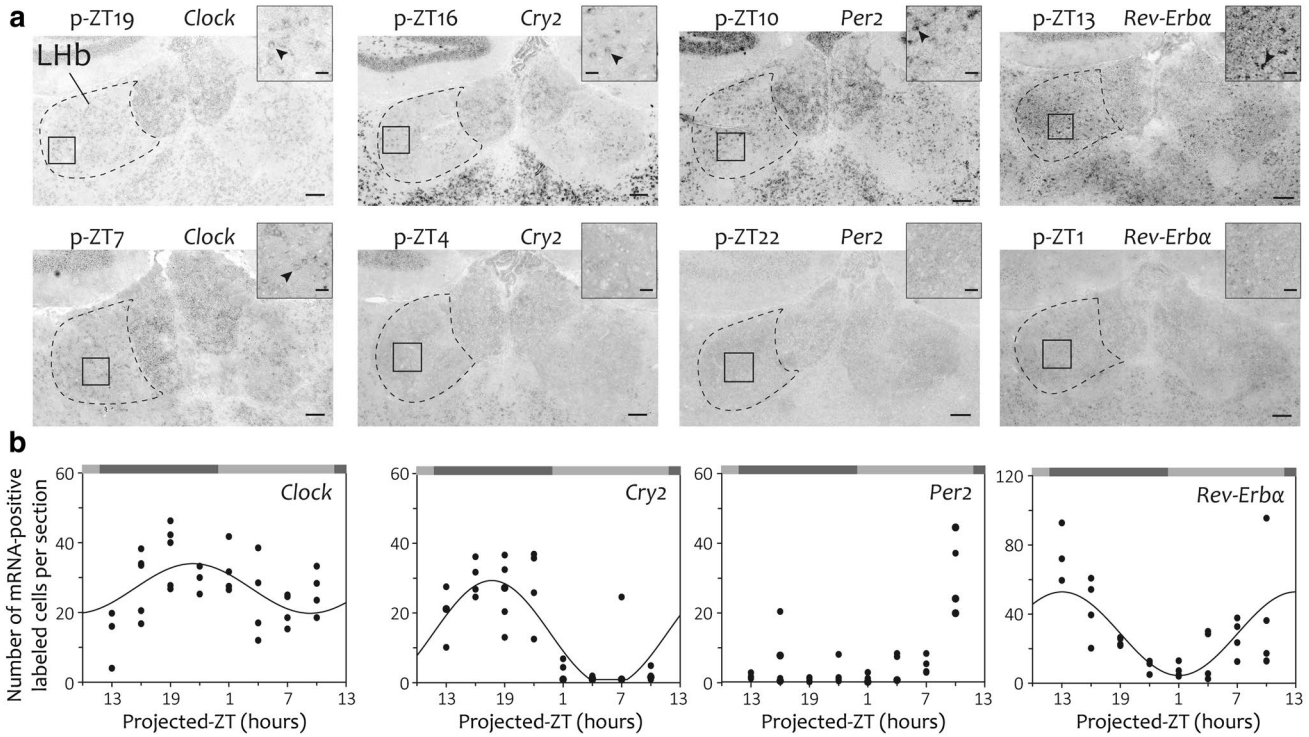

Fig. 1 Circadian gene expression in the LHb of WT mice housed in DD condition for 24 h. **a** Representative photos of the in situ hybridization on coronal brain slices containing the LHb (dashed line) from WT mice using mRNA probes (*Clock, Cry2, Per2* and *Rev-Erbα*). The peak (top row) and the nadir (bottom row) of the expression are represented. Zoomed-in areas correspond to the labeled boxed areas. **b** Number of mRNA-positive labeled cells in the LHb from WT mice. Each closed circle represents an animal and the curves represent the best fit for cosinor analyses. The darkest gray bar on top of each graph represents the subjective night and the lightest gray bar represents the subjective day. *p-ZT* projected-ZT, *ZT Zeitgeber* time. Scale bar: 100 µm for the Hb image; 25 µm for the zoom-in

Table 1 Summary of statistics for the cosinor analysis on the in situ hybridization results from WT animals

	Parameters			Cosinor analysis	
	Mean[a]	Amplitude[a]	Acrophase[b]	Ficher $F_{(2;31)}$	*p* value
Clock	26.89*	7.12*	21.31*	5.2467	0.0114
Cry2	13.49*	15.34*	17.68*	24.1755	< 0.0001
Per2	7.08*	8.75*	10.29*	7.2469[c]	0.0027[c]
Rev-Erbα	28.68*	24.20*	13.02*	12.8704	< 0.0001

*$p < 0.05$

[a]Number of positive cells

[b]In hours (p-ZT)

[c]Did not follow ANOVA rules

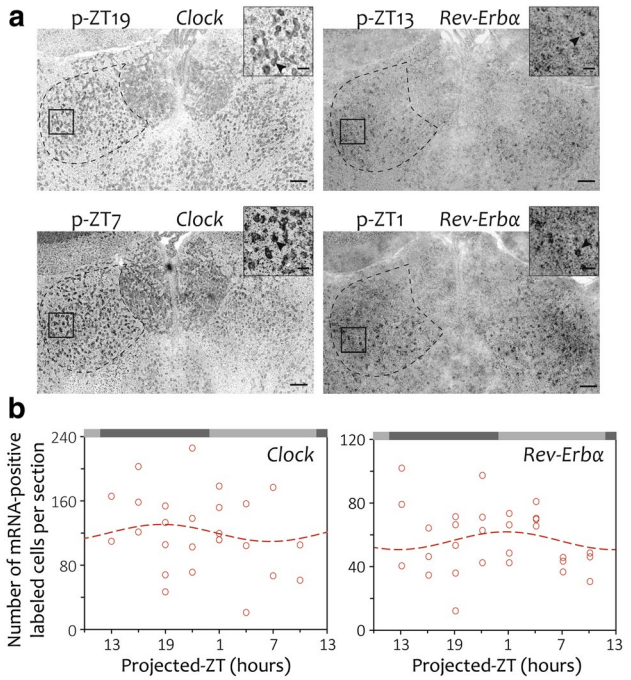

Fig. 2 Circadian gene expression in the LHb of double *Per1*$^{-/-}$–*Per2*^{Brdm1}-mutant mice housed in DD condition for 24 h. **a** Representative photos of the in situ hybridization on coronal brain slices containing the LHb (dashed line) from double *Per1*$^{-/-}$–*Per2*^{Brdm1}-mutant mice using mRNA probes (*Clock* and *Rev-Erbα*). **b** Number of mRNA-positive labeled cells in the LHb from KO mice. Each opened circle represents an animal and the curves represent the best fit for cosinor analyses. The darkest gray bar on top of each graph represents the subjective night and the lightest gray bar represents the subjective day. *p-ZT* projected-ZT, *ZT Zeitgeber* time. Scale bar: 100 µm for the Hb image; 25 µm for the zoom-in

Clock (Fig. 2a, b; Table 2). *Cry2* was non-detectable in *Per* mutant mice (Fig. S2; Table 2). Thus, integrity of *Per* genes is required for clock oscillations in the LHb.

Table 2 Summary of statistics for the cosinor analysis on the in situ hybridization results from KO animals

	Parameters			Cosinor analysis	
	Mean[a]	Amplitude[a]	Acrophase[b]	Ficher $F_{(2;31)}$	*p* value
Clock	110.45*	10.63*	18.66*	0.2751	0.7620
Rev-Erbα	55.43*	5.51*	12.75*	0.5015	0.6114

*$p < 0.05$

[a]Number of positive cells

[b]In hours (p-ZT)

SCN lesions do not interrupt the clock in the Hb

To evaluate to which extent the SCN circadian clock is necessary to sustain circadian oscillations in the Hb, we performed SCN lesions (SCNX) in transgenic *Per2*^{Luc} mice. Behavioral and histological verification indicated that intact control (SHAM) mice showed a rhythmic free-running pattern of locomotor activity with an endogenous period of 24.19 ± 0.05 h (Fig. 3a, c). SCNX mice exhibited a total loss of rhythmic locomotor activity (Fig. 3b, c). Histological verification confirmed complete and successful SCN ablations (Fig. 3d, e). Five SCNX animals which retained rhythmic activity were removed from the analysis.

Unlike for hybridization experiment, we were not able to separate the lateral from the medial Hb; hence the entire Hb clock was investigated. We observed that 9/9 (100%) rostral (Hbr) and 7/9 (78%) caudal (Hbc; Fig. 4c) Hb explants from SHAM animals showed PER2::LUC oscillations with respective periods of 28.10 ± 0.59 and 26.47 ± 1.02 h (Fig. 4a–d). Importantly, in arrhythmic animals with complete SCN lesion, 7/9 (78%) Hbr and 9/9 (100%) Hbc samples displayed sustained PER2::LUC oscillations with respective periods of 28.41 ± 0.61 and 27.27 ± 0.68 h (Fig. 4b, d). Periods did show no significant difference between groups (SHAM vs. SCNX, $F_{(1,31)} = 0.656$; $p = 0.43$) or between areas (rostral vs. caudal, $F_{(1,31)} = 4.068$; $p = 0.053$) or any significant interaction between area and group ($F_{(1,31)} = 0.122$; $p = 0.73$). Because the SCN might be necessary to synchronize clock cells in the Hb, we evaluated the amplitude and phase of the Hb oscillations. The amplitudes in both the Hbr (0.66 ± 0.10 cps in $\log_{10}$) and Hbc (0.65 ± 0.14 cps in $\log_{10}$) of SHAM animals were not significantly different from those of SCNX mice (Hbr, 0.84 ± 0.13 cps; Hbc, 0.60 ± 0.12 cps in $\log_{10}$; $F_{(1,31)} = 0.30$; $p = 0.59$; Fig. 4e). Two-way ANOVA revealed no effect of area ($F_{(1,31)} = 1.166$; $p = 0.29$), and no interaction between area and group ($F_{(1,31)} = 0.807$; $p = 0.38$; Fig. 4e). Amplitude raw data are available in the supplemental Table S1. Moreover, the damping rate of Hb PER2::LUC oscillations was not affected by SCN lesions (Fig. S3).

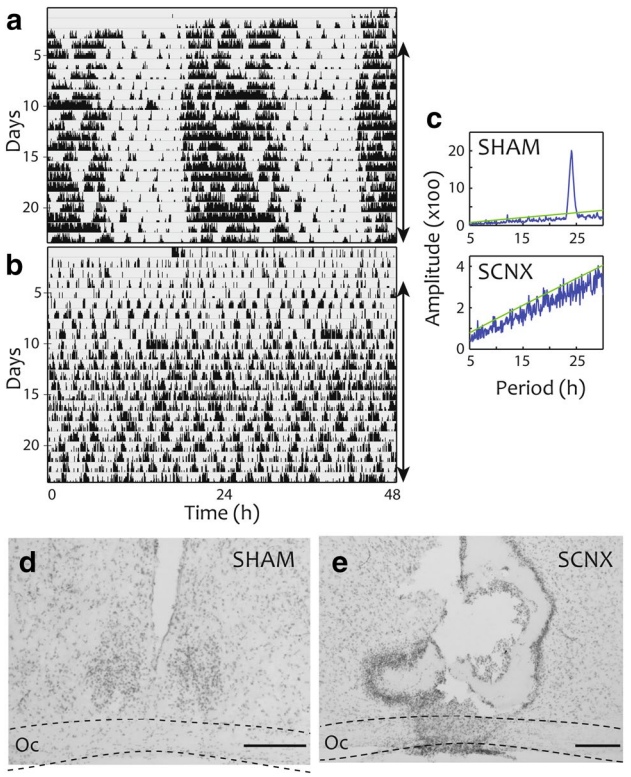

Fig. 3 SCNX histology and behavioral verification. Double-plotted actograms of representative SHAM (**a**) and SCNX (**b**) mice kept in DD condition. SHAM animal showed a circadian rhythm of locomotor activity with a 24 h-superior period, while SCNX mouse was totally arrhythmic. **c** Up-down arrows in **a** and **b** show the 20 days used to determine the period of circadian rhythms of locomotor activity using the χ^2 periodogram method. Cresyl violet staining of coronal sections at the level of the SCN from SHAM (**d**) and SCNX (**e**) mice. Scale bar: 100 µm. *Oc* optic chiasma

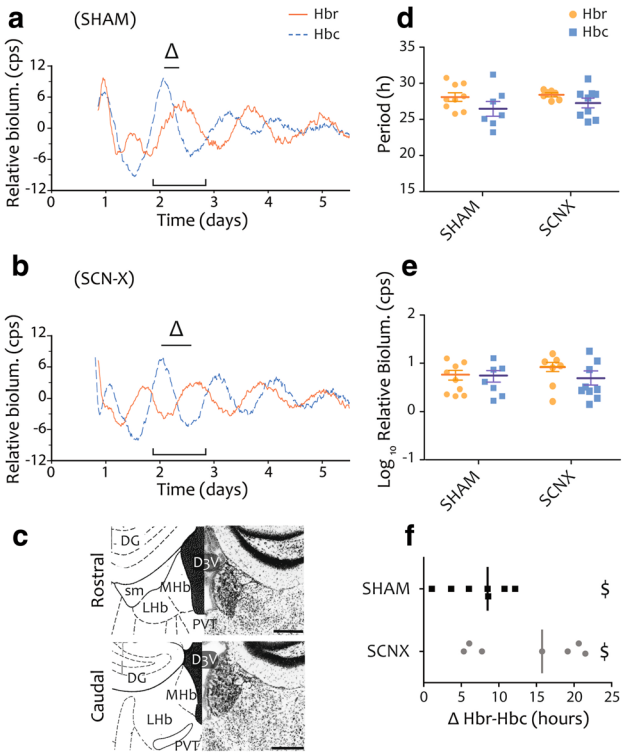

Fig. 4 PER2::LUC circadian oscillations in Hb explants remained stable even after SCN lesions. Hbr (solid orange line) and Hbc (dashed blue line) oscillations from SHAM (**a**) and SCNX (**b**) mice. **c** Schematic and representative pictures (cresyl violet staining) of the two regions of interest of the Hb used for analyses. No difference was noticed in the period (**d**) or in the amplitude (**e**) between Hbr (orange circles) and Hbc (blue squares) explants and between SHAM and SCNX mice. **f** Difference (Δ) between Hbr and Hbc phases. Each phase corresponded to the time when the first peak of PER2::LUC oscillation occurs 24–48 h after the beginning of culture (brackets in **a** and **b** show this time window). Oscillations in the Hbr and Hbc from both SHAM (square) and SCNX (circle) mice were not in phase (different from zero) and were desynchronized (high variance). Scale bar: 250 µm. *Cps* counts per second, *D3V* dorsal 3rd ventricle, *DG* dentate gyrus, *MHb* medial habenula, *LHb* lateral habenula, *PVT* paraventricular thalamus, *sm stria medularis*. $^{\$}p<0.05$, significant difference between the mean and 0. Adapted from Paxinos and Franklin (2001) and Allen Brain Institute (http://www.brain-map.org) mouse atlas

To investigate whether the SCN has a role on the coupling between the Hbr and Hbc clock, we analyzed the Δ phase$_{(\text{Hbr}-\text{Hbc})}$ of PER2::LUC activity. We found that the Δ values in both the SHAM and SCNX groups were significantly different from 0 (respectively $p=0.001$ and $p<0.001$), meaning that Hbr and Hbc oscillations were not in the same phase (Fig. 4a, b, f). Moreover, the phase differences from the SCNX group were more dispersed than those from the SHAM group ($p=0.014$, Levene's test). They were so spread that they could be subdivided into two populations for the SCNX group: one with Δ values around 5 h and the other around 20 h. However, the ANOVA on ranks did not show any difference between the two groups ($p=0.165$).

Lighting conditions affect Hb oscillations

The Hb receives inputs from the retina directly via retinal ganglion cells or indirectly, such as in the *zebrafish*, through a thalamic nucleus (Qu et al. 1996; Reuss and Decker 1997; Hattar et al. 2006; Cheng et al. 2017), and responds to light after photo-stimulation of the retina (Zhao and Rusak 2005; Dreosti et al. 2014; Shuboni et al. 2015; Cheng et al. 2017). Therefore, to unravel how lighting conditions might affect circadian activity of the Hb clock, we exposed two groups of *Per2*^{Luc} mice to either a light-dark cycle (LD) or constant light (LL) condition, and compared rhythms of PER2::LUC bioluminescence with previous results obtained for SHAM animals under DD condition. Mice under LD cycle showed a clear entrained behavioral rhythm with main locomotor activity at night (Fig. 5a, c), whereas mice under LL condition showed a progressive lengthening of the period of locomotor

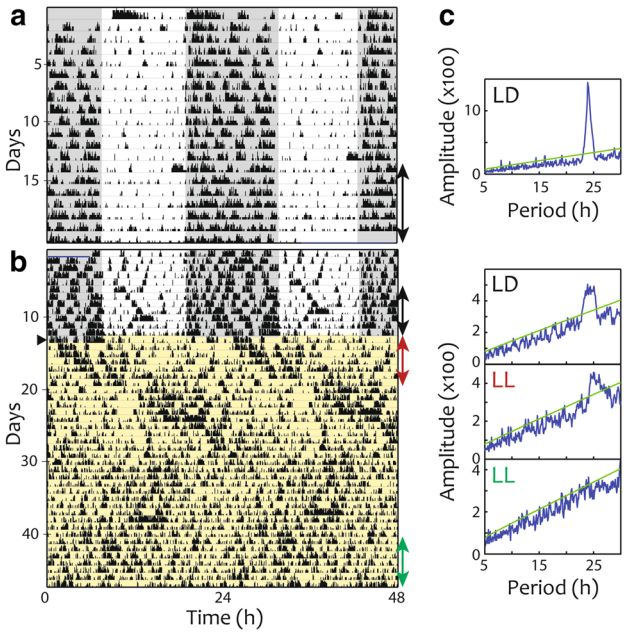

Fig. 5 The LL condition induced loss of circadian rhythms of locomotor activity. Double-plotted actograms of mice kept in LD (**a**) and LL conditions (**b**). At first, animals from both groups (LD or LL) were synchronized to a 12:12 h-LD cycle (black up-down arrows). Under LL exposure (**b**), mice showed a lengthening of the circadian period (red up-down arrow in **b**) and then became arrhythmic (green up-down arrow in **b**). **c** Up-down arrows in **a** and **b** indicate the days used to determine the period of circadian behavior using the χ^2 periodogram method; periodograms follow the same order as arrows

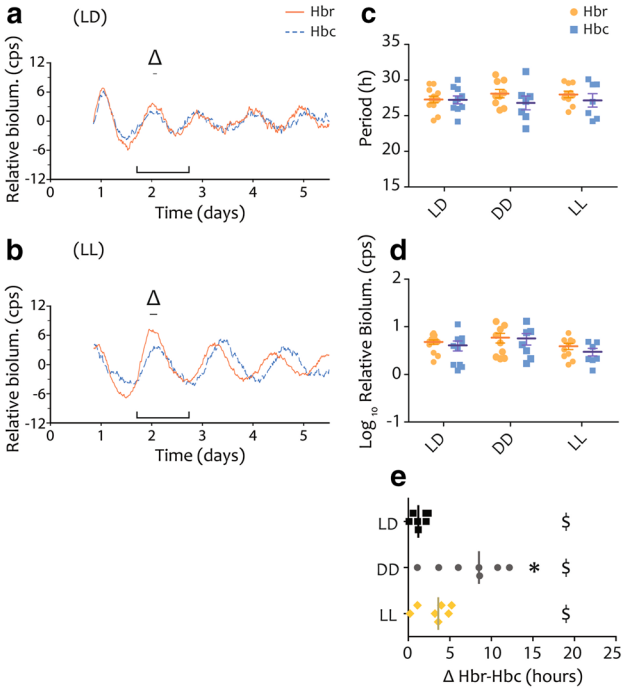

Fig. 6 Constant darkness uncoupled oscillations of Hb explants. Hbr (solid orange line) and Hbc (dashed blue line) oscillations from LD (**a**) and LL-exposed (**b**) mice. No difference was noticed in the period (**c**) or in the amplitude (**d**) between Hbr (orange circles) and Hbc (blue squares) explants and between LD, DD and LL conditions. **e** Δ values between Hbr and Hbc phases according to lighting conditions. Each phase corresponded to the time when the first peak of PER2::LUC oscillation occurs 24–48 h after culture (brackets in **a** and **b** show this time window). Phase differences of Hbr and Hbc were different from zero and those in LD (square) and LL (diamond) conditions were clustered. However, under DD condition, there was a higher and significant variance (desynchronization) between the two parts of Hb compared to the other housing light conditions. *Cps* counts per second. $^{\$}p<0.05$, significant difference between the mean and 0. $^{*}p<0.05$, significant difference vs. LD

activity, and then total loss of behavioral rhythms after 5 weeks of LL exposure (Fig. 5b, c).

In animals exposed to a LD cycle, PER2::LUC oscillations were observed in 12/14 (86%) Hbr and 10/14 (71%) Hbc with respective periods of 27.28 ± 0.47 and 27.23 ± 0.55 h (Fig. 6a, c). In arrhythmic animals exposed to LL condition, PER2::LUC oscillations were maintained with periods of 27.98 ± 0.46 h in 10/12 (83%) Hbr and 27.15 ± 0.94 h in 7/10 (70%) Hbc (Fig. 6b, c). Periods of Hb (rostral and caudal) from animals that were exposed to a LD cycle or to LL condition did not differ from those of animals in DD condition (Figs. 4d, 6c, $F_{(2,49)}=0.139$; $p=0.87$), without difference between regions ($F_{(1,49)}=2.508$; $p=0.12$), or interaction between area and group ($F_{(2,49)}=0.779$; $p=0.46$). Amplitudes of PER2::LUC oscillations did not differ between animals exposed to a LD cycle (Hbr 0.65 ± 0.05 cps; Hbc, 0.50 ± 0.10 cps in $\log_{10}$), LL condition (Hbr, 0.54 ± 0.07 cps; Hbc, 0.42 ± 0.08 cps in $\log_{10}$) or DD condition (Hbr 0.67 ± 0.10 cps; Hbc, 0.64 ± 0.12cps in $\log_{10}$; $F_{(1,49)}=1.69$; $p=0.16$), and there was no difference between regions ($F_{(1,49)}=1.722$; $p=0.20$), or interaction between area and group ($F_{(2,49)}=0.261$; $p=0.77$; Fig. 6d). Amplitude raw data are available in the supplemental Table S1. By contrast, LL exposure led to a significant decrease in amplitude of PER2::LUC oscillations in SCN explants when compared to those from animals under LD cycles (Fig. S4), suggesting that the LL condition was able to affect the SCN.

Interestingly, when we analyzed the Δ phase $_{(Hbr-Hbc)}$ of the PER2::LUC bioluminescence activity, we found that all Δ values in the different lighting conditions were different from 0 (LD $p=0.003$; DD $p=0.001$; LL $p=0.002$), suggesting that Hbr and Hbc were not perfectly in phase (Fig. 4a, 6a, b, e). However, the DD condition appeared to affect the Hb clock in a distinct way since Δ phases were more dispersed than those of LD and LL groups ($p=0.0036$, Levene's test). The ANOVA on ranks showed an effect of group ($p=0.014$) and pointed to the difference between Δ phase$_{(Hbr-Hbc)}$ from LD and DD conditions ($p=0.014$). Together, phase differences suggest that lighting information is important for coupling of Hbr and Hbc oscillators. It is important to note

that acrophases in Hb explants from animals kept in LD or DD were not under the influence of the time of sacrifice (Fig. S5).

Discussion

In the present study, we reported that the rostral and caudal regions of the Hb oscillate robustly with similar period and amplitude independently from the SCN and the lighting conditions. However, despite the ability of the two parts of the Hb to oscillate robustly in a circadian manner, they lose their coupling in the absence of light cues even with an intact SCN (SHAM group in DD condition). We also observed that the circadian expression of clock genes in the Hb is altered in double *Per1*$^{-/-}$*–Per2*^{Brdm1}-mutant mice, suggesting that despite the independence from the SCN, the Hb clock needs the clock genes to keep circadian timing.

Rhythmic activity of the Hb has been previously reported for different parameters: for instance, in the daily profile of electrophysiological activity of both the LHb and medial Hb (Sakhi et al. 2014a, b), in clock genes and protein expression, and c-Fos expression in the LHb of animals under LD condition (Shieh 2003; Tavakoli-Nezhad and Schwartz 2006; Zhao et al. 2015; Christiansen et al. 2016). Here, unlike in previous studies, animals were kept under DD condition to explore the endogenous rhythmicity (i.e., clock gene expression and PER2::LUC oscillations) of the Hb clock, independently of the LD cycle. In vivo, we observed that expression of *Cry2, Clock* and *Rev-Erbα* clock genes was rhythmic in the LHb of animals maintained in DD condition. We found that the LHb cells expressed *Per2* gene for a short time around the end of subjective day (pZT10). Similarly, in rats under LD cycle, both the *Per2* mRNA and protein are expressed rhythmically and peak at day time (ZT6-10) in the LHb (Zhao et al. 2015). Thus, the short delay on the peak of expression in our study might reflect the free-running expression of animals under DD condition with a period superior to 24 h. The narrow window of expression in our experiment might be also explained by the method of analysis: we counted the number of positively labeled cells instead of quantifying labeling intensity. Unlike what has been reported for the SCN, the cosinor analysis revealed that *mClock* mRNA showed rhythmic expression as assessed by the number of labeled cells in the LHb. Thus, the LHb, such as the pineal gland, presents circadian variations in the expression of the *Clock* gene (Karolczak et al. 2004). Accordingly, a daily variation of expression of the CLOCK protein has been also found in other brain areas such as the arcuate nucleus, paraventricular thalamus, dorsomedial hypothalamus, paraventricular nucleus and dentate gyrus (Wyse and Coogan 2010). Our result also fits with the observation that CLOCK and BMAL1 proteins display rhythmic expression in the LHb in LD condition (Wyse and Coogan 2010).

We also found circadian rhythms in the expression of *Rev-Erbα* and *Cry2* genes in the LHb, with acrophase occurring at the early (pZT13) and mid-subjective night (pZT17), respectively. REV-ERBα is a key clock factor with transcriptional repression activity and it is expressed rhythmically in the SCN with an acrophase during the day (Preitner et al. 2002). Here, to our knowledge, this is the first time that rhythmic expression of *Rev-Erbα* is reported in the LHb. If it has a similar role as in the SCN, thus we should observe a repressive action on its principal targets (*Bmal1* and *Clock*). At the clockwork level, *Rev-Erbα* is induced by the CLOCK/NPAS2:BMAL1 heterodimer, and then REV-ERBα, in turn, represses the expression of these three transcription factors (Preitner et al. 2002; Crumbley and Burris 2011; Ripperger and Albrecht 2012). Here, rhythmic expression of *Clock* gene was in antiphase with that of *Rev-Erbα*, suggesting that the negative control of REV-ERBα over *Clock* also exists in the LHb. Interestingly in double *Per1*$^{-/-}$*–Per2*^{Brdm1}-mutant mice, the expression of *Clock* and *Rev-Erbα* mRNA was arrhythmic and that of *Cry2* was undetectable. A previous study showed that single mutation in *Per2* gene resulted in significant decrease of *Cry1, Per1* and *Bmal1* clock gene expression (Bae et al. 2001). The double *Per1-2* mutation may worsen the phenotype in our study to the point of making the *Cry2* mRNA undetectable. Our results are consistent with those from the study of Sakhi et al. (2014b) which showed that the double knock-out of *Cry1-2* genes abolishes the *Per1-Luciferase* rhythmic activity in the mouse Hb. This suggests that malfunction of the molecular clock disrupts rhythmic expression of clock genes in the LHb.

Since the generation of the *Per2*^{Luc} transgenic mouse model, the identification of organs and brain regions with rhythmic activity has been extensively explored (Yoo et al. 2004; Prolo et al. 2005; Ruan et al. 2008; Guilding et al. 2009, 2010; Leise et al. 2012; Jaeger et al. 2015; Besing et al. 2017; Brancaccio et al. 2017). This mouse model has been used to demonstrate sustained circadian oscillations in the LHb (Guilding et al. 2010). Here, we confirm the results from Guilding et al. (2010), but report increased sustainability of PER2::LUC bioluminescence activity. This could be explained by the augmented thickness (500 µm instead of 300 µm) of our samples.

Because our aim was to decipher whether the Hb clock is dependent or not on the SCN master clock, we monitored PER2::LUC activity in the Hb from arrhythmic SCN-lesioned animals. We did not observe changes in period or amplitude of PER2::LUC oscillations of Hb explants from arrhythmic SCN-lesioned animals, nor from arrhythmic animals by LL condition, suggesting that the Hb clock does not require the SCN pacemaker to maintain its own oscillations. Indeed, LL exposure was reported to affect the

SCN by downregulating the amplitude of the PER2::LUC and *Per1-luciferase* activity, and to alter the phase relationship between the SCN and other circadian clocks (present results; Granados-Fuentes et al. 2004; Ohta et al. 2005). This has been suggested to be the result of the desynchronization of multiple SCN oscillators (Ohta et al. 2005). Unlike the SCN, the Hb and the olfactory bulb (OB) kept circadian time in LL condition (Granados-Fuentes et al. 2004). Melanopsin-expressing retinal ganglion cells (ipRGC) projecting to the SCN and Hb differ in density and localization (direct vs. indirect innervations). Whereas in the SCN, they arrive directly to the nucleus in its ventral part, for the Hb the iPRGCs target mainly the PHb region (Hattar et al. 2006; Fernandez et al. 2018). The existence of this relay might explain differences observed in the amplitude of PER2 oscillations between explants (the SCN vs. the Hb) of animals exposed to LL condition (Hattar et al. 2006; Fernandez et al. 2018). However, these analyses are from ex vivo experiments. Thus, bioluminescence studies in freely moving mice may underline other characteristics of the Hb clock or the involvement of the SCN in the Hb oscillations (Hamada et al. 2016).

In a previous study of Xu et al. (2015) in rats, SCN lesions altered the day–night expression of *Per2* and *c-fos* in the Hb. Different to our study, authors evaluated *c-fos* and *Per2* mRNA expression at only two time points (day vs. night), which is not sufficient to conclude that the Hb lost rhythmic activity, and rather underwent a phase shift. Indeed, the coupling between the two Hb parts of the same animal has been revealed in our study. First, in LD cycle, phase differences were quite close to zero and clustered, meaning that the Hbr and Hbc did not peak exactly at the same time but they stayed in synchrony. Similarly, these two oscillators kept their phase relationship under LL exposure. Nevertheless, large dispersion of Δ values was observed in DD condition and even more in arrhythmic SCN-lesioned animals, suggesting that in the absence of the principal synchronizer for the circadian system (light), coupled clocks in the Hb become internally desynchronized and even more if the main pacemaker (the SCN) is also missing. This suggests that the external input (light) for the Hb clock seems more important than the internal signal (SCN). Moreover, under DD condition, the free running of the SCN and Hb PER2 rhythm, with a different period, may cause a more pronounced uncoupling between them, and consequently between the clocks in the Hbr and Hbc.

Even if the presence of some fibers originating in the retina have been described in the Hb (Qu et al. 1996; Reuss and Decker 1997), the ipRGCs project to the PHb region which might be important as a relay to maintain the coupling between the Hbr and Hbc oscillators by light stimulus (Hattar et al. 2006; Fernandez et al. 2018). Moreover, the PHb might modulate light information precluding overstimulation of the Hb, and therefore, keeping the Hb nuclei coupled in LL condition. By contrast, Hb's nuclei might get into free run in DD condition and thus loose synchrony. In *zebrafish*, a thalamic nucleus acting as a relay between the retina and Hb has been reported (Cheng et al. 2017; Lin and Jesuthasan 2017). Therefore, other potential candidates, beyond the PHb, as thalamic or hypothalamic nuclei (which receive ipRGC innervations; Hattar et al. 2006; Zahm and Root 2017) could be also found in rodents as important relay structures to transmit light information to the Hb. Further in vivo recordings of PER2::LUC activity in the Hb from animals exposed to LL condition or to flashing lights should confirm the functional link between the retina and the Hb clock (Hamada et al. 2016). Interestingly, the double *Per2*/*Cry1* mutant or the vasoactive intestinal peptide (VIP) receptor mutant mice are arrhythmic in DD, while their behavioral rhythms are rescued upon exposure to LL condition (Abraham et al. 2006; Hughes et al. 2015). This situation may reflect the fact that behavioral and physiological perturbations might appear when the (circadian) system is lacking of external signals, leading to uncoupling of clocks within the same structure.

The presence of different oscillators along the rostro-caudal axis was previously reported in the SCN (Jagota et al. 2000; Honma et al. 2012). These oscillators show two independent peaks of the firing rate activity: a morning peak at dawn and an evening peak at dusk, which have been suggested as the morning and evening coupled oscillators encoding photoperiod (Jagota et al. 2000; Pittendrigh and Daan 1976). Here, we reported the presence of two mutually coupled oscillators in the Hb which are in the rostro-caudal axis. What is the functional relevance of these oscillators is, however, unknown, but these might have a similar role in the differential integration and regulation of external or internal time signal. Few studies have evaluated the anatomical differences along the rostro-caudal axis of the Hb (Zhang et al. 2016, 2018). According to these results, Hb oscillators may differ in the circadian regulation of behaviors such as food or water intake. However, this hypothesis remains to be tested.

The Hb, and particularly the LHb, is also an important nucleus implicated in the regulation of motivational and emotional states (Mizumori and Baker 2017), because it modulates the monoamine system (mainly dopamine and serotonin) in the midbrain (Namboodiri et al. 2016). Interestingly, long time exposure to DD condition results in important damage to the monoamine system (serotonin from the raphe nuclei and dopamine from the VTA) and depressive-like behavior in rats, which are reversed when animals are treated with antidepressant drugs (Gonzalez and Aston-Jones 2008). Whether uncoupling of the two regions (rostral and caudal) of the Hb might be part of the mechanism whereby constant darkness could affect mood, is not known. Hence, it may be interesting to evaluate monoamine and behavioral

changes in animals with uncoupled Hb clock(s). Importantly, in a previous study, it was reported that noradrenaline (NA) concentrations are different between the rostral and caudal Hb areas: higher concentrations were found in the rostral than the caudal part. Indeed, the rostral Hb receives sympathetic and central NA innervations, and the caudal Hb receives only central innervations (Gottesfeld 1983).

Conclusion

In the present study, we demonstrate the importance of entrainment signals (e.g., light, SCN timing) for the Hb clocks to keep coupled, and the relevance of *Per* genes to maintain LHb circadian activity. Moreover, whereas constant LL does not appear to affect the Hb clock, the lack of light/dark alternations (e.g., DD) desynchronizes at least two possible oscillators present in the Hbr and Hbc. Internal desynchronization of the circadian system (into the SCN, Hb or between circadian clocks) has been proposed as part of the consequences induced by different circadian perturbations in human conditions such as jet-lag or shift-work, and which leads to psychiatric pathologies (Karatsoreos 2014). Therefore, understanding the clock mechanisms in the Hb will allow a better comprehension of its involvement in physiology (reward, motivation) and physiopathology (drug intake, mood).

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Compliance with ethical standards

Conflict of interest Authors declare no potential conflicts of interest.

Human or animal rights All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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